

# Assessment of Antimicrobial activity of *Cajanus cajan* leaves against selected strains of bacteria and fungi

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## ABSTRACT

There is a pressing demand for alternative therapeutic agents made from natural sources due to the rising incidence of antibiotic resistance. A promising source of bioactive substances with possible antibacterial and antifungal qualities is found in medicinal plants. Utilizing methanol, acetone, and petroleum ether as solvents, the researchers employed agar well diffusion and minimum inhibitory concentration (MIC) assays to assess the extracts' effectiveness against both bacterial pathogens (*Staphylococcus* species and *Escherichia coli*) and fungal pathogens (*Candida* and *Aspergillus* species). Results indicated that all solvent extracts demonstrated antimicrobial activity, though efficacy varied by solvent polarity, type of microorganism, and concentration. The methanol extract was particularly effective, yielding the largest zones of inhibition against *Staphylococcus* and significant activity against *E. coli*, similar to standard antibiotics. The acetone extract showed moderate effectiveness, while petroleum ether demonstrated lower inhibition, underscoring the correlation between solvent polarity and extraction of antimicrobial compounds. In conclusion, leaf extracts from *Cajanus cajan*, especially the methanolic fraction, have strong antibacterial qualities and could be a source of natural antimicrobial agents. To investigate their medicinal applications, more research on separation, purification, structural characterization, toxicity assessment, and mechanism of action studies is advised. In addition to providing safer and more affordable treatment options, the development of plant-based antimicrobials may help fight antimicrobial resistance.

**Keywords:** *Cajanus cajan*, antimicrobial assay, *Staphylococcus*, *E. coli*, *Aspergillus*, *Candida*, and antimicrobial resistance.

## 1. Introduction

The rise of antimicrobial drug resistance and the frequent incidence of unfavorable side effects of various antibiotics have made the hunt for plants with antibacterial activity more crucial in recent years. *Cajanus cajan* L., sometimes referred to as "arhar" in Hindi and "pigeon pea" or "redgram" in English, is a perennial member of the Leguminosae family. It is one of the most significant legume crops for food, and it is mostly farmed in tropical areas. About 90% of the world's production of *Cajanus cajan* L. comes from India. While pigeon pea is only ranked sixth in terms of area and production when compared to other grain legumes, it is utilized in a wider variety of ways [1]. It is a hairy, upright shrub that grows to a height of one to two meters. The leaves have three leaflets and range from oblong-lanceolate to oblanceolate. The yellow flowers are about 1.5 cm long and come in sparsely spaced, peduncled racemes. The hairy pod is 4–7 cm long and 1 cm broad, with two–seven seeds within. It has a lot of proteins. In India, the leaves are used to raise silkworms, the green pods are consumed as a vegetable, and the husk, green leaves, and tops are used as green manure and fodder. Among its several medical applications, *C. cajan* is recommended as a sedative and for the treatment of pain in traditional Chinese medicine [2]. Many researchers throughout the world have looked into the antibacterial qualities of plants, but careful biological testing of plant extracts is necessary to guarantee their effectiveness and safety. If plant extracts are to be recognized as legitimate therapeutic agents for the treatment of infectious illnesses, these elements are crucial [3].

According to studies on chemical constituents, pigeon pea leaves are high in flavonoids and stilbenes, which are thought to

be beneficial to human health. Finding novel antimicrobial compounds from alternative sources, including plants, has become essential in the current situation of multidrug resistance to human pathogenic illnesses. Flavonoids, stilbenes, saponin, tannin, reducing sugars, resins, and terpenoids—which may have antioxidant, antibacterial, hypocholesterolemic, and anti-inflammatory properties—are abundant in *C. cajan* leaves. *C. cajan* seeds are utilized as food and animal feed because they are high in proteins, minerals, and unsaturated fatty acids. Additionally, *C. cajan* seeds are used as an antimalarial treatment and to treat diabetes, fever, diarrhoea, hepatitis, and measles [4]. Even with these encouraging results, nothing is known about the phytochemical makeup of *C. cajan* outside of Asia and Africa. Geographical location, soil composition, plant cultivar, and farming techniques can all affect the concentration and profile of its bioactive components. Nevertheless, *C. cajan* in Panama and Latin America has not been well studied [5].

Among the most common and extensively dispersed families of plant metabolites are phenolic compounds. Anti-apoptosis, antiaging, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection, improved endothelial function, angiogenesis, and reduction of cell proliferation are some of their biological characteristics [6]. Studies carried out in Nigeria, China, and India have shown the antibacterial activity of ethanolic and methanolic extracts of *C. cajan* leaves, seeds, and roots, building on the plant's medicinal qualities.

These extracts have shown antimicrobial activity against various microorganisms, including *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus sp.*, *Salmonella thypi*, *Klebsiella sp.*, and *Escherichia coli* [7].

## 2. Materials and Methods

**2.1 Plant Collection and identification:** The pathogen-free and healthy leaves of *Cajanus cajan* were collected from the Viqarabad region of Telangana. The plant has been authenticated by the Department of Botany, Anwarul Uloom College, Hyderabad. The plant material was washed thoroughly first with tap water and then distilled water to remove the surface contaminants. Later, they are shade-dried for about two weeks at room temperature to remove the moisture content and then pulverized using a laboratory blender. The powder should be stored in an air-tight container.

**2.2 Plant Extract Preparation:** Each of three conical flasks containing 30 grams of dried leaf powder is filled with 200 milliliters of methanol, 200 milliliters of acetone, and 200 milliliters of petroleum ether. After that, it is placed on the magnetic stirrer and wrapped in aluminum foil. With medium stirring, the stirrer's temperature is kept between 25 and 30 degrees Celsius. In this situation, the extraction procedure lasts for almost 72 hours. After that, the extracts are filtered using Whatman Filter Paper No. 1. The rotatory evaporator is used to concentrate the filtered extracts, which may then be stored at the ideal temperature for later use.

**2.3 Qualitative Analysis:** *Cajanus cajan* leaves extracts were screened for bioactive components using a number of chemical assays. According to Trease and Evans' established protocols, the tests were conducted (2002) [8].

**2.3.1 Test for Flavonoids:** Flavonoids were detected by adding a few drops of 20% sodium hydroxide to 2 milliliters of each extract, which produced a bright yellow hue. After adding a few drops of 70% diluted hydrochloric acid, the yellow hue vanished. The presence of flavonoids in the sample extract is shown by the production and disappearance of yellow.

**2.3.2 Test for Tanins:** After boiling 200 mg of the plant extract with 10 mL of distilled water and adding 0.1% ferric chloride, the liquid was checked for the presence of tannins by looking for a blue-black hue [9].

**2.3.3 Test for Alkaloids:** Two milliliters of the filtrate and three drops of 1% HCl were used to cook the plant extract in steam after it had been dissolved in one hundred milliliters of water. Next, 6 mL of the Mayer-Wagner reagent was mixed with 1 mL of the heated mixture. Alkaloids were present when a cream or brown-red precipitate appeared [9].

**2.3.4 Test for Steroids:** Concentrated H<sub>2</sub>SO<sub>4</sub> and 2 milliliters of chloroform were added side by side to the crude extract. The presence of steroids is shown by the development of red in the bottom chloroform layer. Another experiment was carried out in which crude extract was combined with two milliliters of chloroform. Next, two milliliters of concentrated H<sub>2</sub>SO<sub>4</sub> and two milliliters of acetic acid were added to the mixture. The presence of steroids in the sample is indicated by its greenish appearance [10].

**2.3.5 Test for Glycosides:** Two milliliters of glacial acetic acid were combined with the crude extract after a few drops of a 2% FeCl<sub>3</sub> solution were added. After that, the mixture was moved to another container with two milliliters of concentrated H<sub>2</sub>SO<sub>4</sub>. The presence of cardiac glycosides in the sample is shown by the development of a brown ring at the interface [10].

**2.3.6 Test for Terpenoids:** Terpenoids were detected using the Salkowski technique. After combining extract (5 ml) with chloroform (2 ml), a layer of strong sulfuric acid (3 ml) was carefully applied. The interface developed a reddish-brown coloring to indicate the presence of terpenoids [11].

**2.3.7 Test for Saponins:** Twenty milliliters of distilled water were added to a test tube containing one milliliter of the stock solution. For fifteen minutes, it was manually shook. The test tube's top developed a layer of foam. The presence of saponins was suggested by this layer of foam [12].

**2.3.8 Test for Phenols:** In a test tube, one milliliter of plant extract was dissolved in one milliliter of sterile distilled water. Three drops of 1% (w/w) FeCl<sub>3</sub> were added to the mixture. Polyphenols and tannins are indicated by the blue-black/violet hue. Another lead acetate test was carried out in which a test tube was filled with the plant extract. The combination was mixed with 3 milliliters of a 10% lead acetate solution. The presence of phenolic compounds is shown by the formation of a white precipitate [13].

## 2.4 Antimicrobial Assessment:

**2.4.1 Preparation of active bacterial cultures and test sample concentrations:** A single bacterial colony of pure culture is transferred into a 150ml conical flask containing 50ml nutrient broth media and incubated for 8-12hrs at 37 °C. For the MIC test, samples of powdered compounds were dissolved in 1 milliliter of an appropriate solvent, such as water, methanol, or DMSO, and then divided into aliquots of varying concentrations. Depending on the necessary concentrations, liquid samples were utilized directly and diluted with water or a solvent.

**2.4.2 Antibacterial Assay:** The pour plate technique, which involves mixing 1% of active bacterial cultures into autoclaved agar medium shortly before solidifying temperature and pouring the mixture into the plates, was used to perform the antibacterial test. In this case, the Gram positive and Gram negative bacteria were *Staphylococcus* and *E. coli*, respectively. Following the solidification of the plates, wells were created using a sterile well borer, and 100µl of each sample was placed into each well. Plates were kept in a bacterial incubator for 18–24 hours at 37 °C. The clear inhibition zone, also known as the zone of clearing, is measured from one end to the other. The substance has reduced inhibitory effect if the growth is good (less mm), and strong inhibitory activity if the growth is poor (more mm). The sizes of the inhibitory growth zones are typically evaluated after the antimicrobial agent diffuses into the agar and prevents the test microorganism from germinating and growing [14].

**2.4.3 Minimum inhibitory concentration (MIC) assay for antibacterial property of given samples:** By loading 25 µl, 50 µl, 75 µl, and 100 µl of samples diluted with water/solvent to make up the amount up to 100 µl in each well of the plate, the samples that had been tested for antibacterial activity were

assessed for their minimum inhibitory concentrations (MIC). Samples containing 10 mg/mL were utilized in this experiment, and the appropriate dilutions were prepared. The MIC value was calculated by evaluating the phytopathogens' apparent growth [15].

**2.4.4 Antifungal Assay:** *Aspergillus* and *Candida* were used in the antifungal assessment. Yeast extract peptone agar and potato dextrose agar were made and autoclaved. To prevent bacterial contamination, an antibiotic (Streptomycin/ Chloramphenicol) was added to the medium right before it was poured onto the plates. After allowing the plates to set, 5 mm wells were created using a sterile well borer according to the quantity of samples. Each well held 100µl of the samples. The plant extracts were allowed to freely diffuse in the agar for five to ten minutes. The plates were sealed with paraffin tape and incubated for 96 hours at 25 °C while inverted. A transparent scale that has been calibrated was used to quantify the antifungal activity in millimeters (mm) [16].

**2.4.5 Minimum inhibitory concentration (MIC) assay for antifungal property of given samples:** The minimum inhibitory concentrations (MIC) of the antifungal activity-checked samples were determined by loading 25 µl, 50 µl, 75 µl, and 100 µl of samples diluted with water or solvent to make up the volume up to 100 µl in each well of the plate, respectively. Samples containing 10 mg/mL were utilized in this experiment, and the appropriate dilutions were prepared. The minimum inhibitory concentration (MIC) of the plant extract was defined as the lowest concentration at which no colour change occurred [17].

### 3. Results and Discussion

**3.1 Qualitative Analysis:** The qualitative analysis of methanol, acetone and petroleum ether extracts of the leaves of *Cajanus cajan* revealed the presence of various secondary metabolites. The presence or absence of phytochemicals was confirmed by visual observation of the change in color or turbidity [18]. The table 1 shows the results of phytochemical screening in all the three extracts.

Table 1: Phytochemical analysis of *Cajanus cajan*

S.No.	Phytochemical Constituents	Methanol Extract	Acetone extract	Petroleum ether extract
1	Flavonoids	+	+	--
2	Tannins	+	+	+
3	Alkaloids	+	+	--
4	Steroids	+	+	+
5	Glycosides	+	+	+
6	Terpenoids	+	--	--
7	Saponins	+	+	+
8	Phenols	+	+	+

### 3.2 Antimicrobial Assessment of the *Cajanus cajan* leaf extract

**3.2.1 Zone of inhibition:** Compared to the conventional antibiotic (14 mm), the methanol extract showed the strongest antibacterial activity, especially against *Staphylococcus* (16 mm). This implies that bioactive antimicrobial chemicals may be effectively extracted using methanol as a solvent. In comparison to the usual medication, the acetone extract shown modest action (14 mm) against both bacterial strains. The non-polar portion included fewer or weaker antibacterial chemicals, as evidenced by the petroleum ether extract's lowest activity (12 mm). Although efficacy varied with solvent polarity, all extracts showed action against both Gram-positive (*Staphylococcus*) and Gram-negative (*E. coli*) bacteria, indicating broad-spectrum potential.

Table 2: Zone of inhibition for antibacterial assay

S.no	Sample ID	Sample Name	Bacterial pathogens	
			Zone of inhibition (mm)	
			<i>Staphylococcus</i>	<i>E. coli</i>
1	1	Methanol Ext.	16mm	14mm
2	2	Acetone Ext.	14mm	14mm
3	3	Petroleum Ether Ext.	12mm	12mm
4	Standard	Streptomycin	14mm	14mm



Fig 1: Zone of inhibition for antibacterial assay

**3.2.2 Minimum inhibitory concentration (MIC) assay for antibacterial property of given samples:** The zone diameter increase from 8 mm to 16 mm as the concentration of the methanol extract increased, with inhibition beginning at 50 µL. Strong antibacterial potency and concentration-dependent action are indicated by this. In comparison to the methanol extract, the acetone extract only showed inhibition at 75 µL and higher, indicating modest antibacterial activity. Although the zones of inhibition were smaller, the petroleum ether extract still demonstrated inhibition at 75 µL, suggesting that the non-polar extract contained relatively weaker antimicrobial chemicals. The findings unequivocally show that antibacterial activity rises with concentration, supporting phytochemicals' dose-dependent action against *Staphylococcus*.

Table 3: MIC for *Staphylococcus*

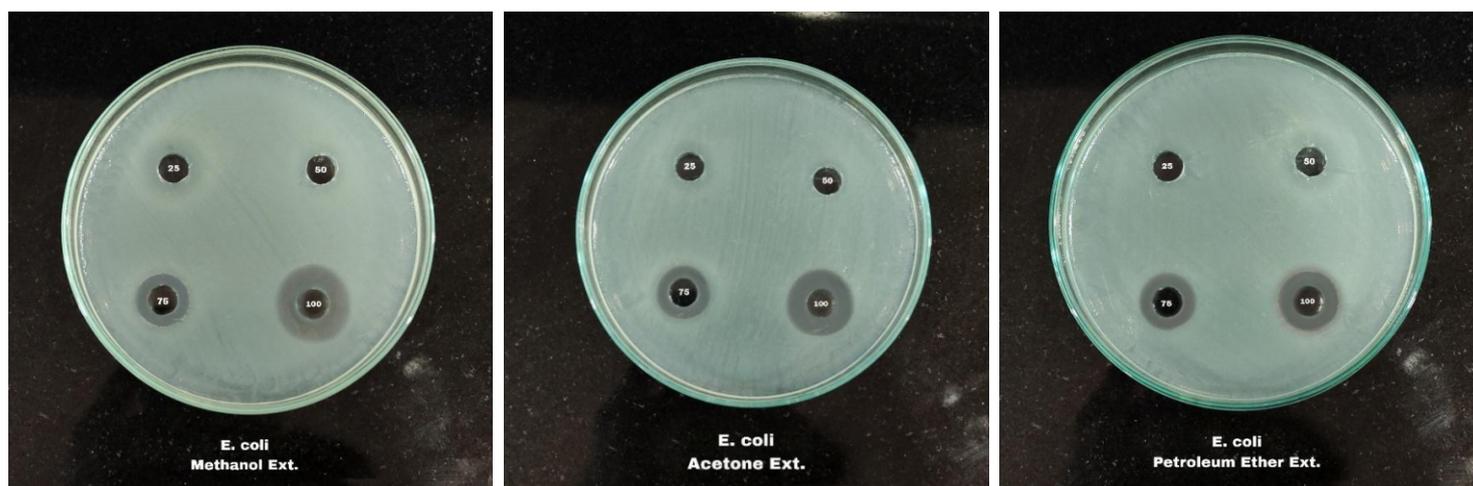
S.No	Sample ID	MINIMUM INHIBITORY CONCENTRATION – MIC (mm)				MIC of sample (µL)
		<i>Staphylococcus</i>				
		25µL	50µL	75µL	100µL	
1	Methanol Ext.	-	08mm	12mm	16mm	50µL
2	Acetone Ext.	-	-	12mm	14mm	75µL
3	Petroleum Ether Ext.	-	-	08mm	12mm	75µL

Fig:2 Minimum inhibitory concentrations of *Staphylococcus*

Starting at 75 µL, all three extracts had antibacterial action against *E. coli*, suggesting that the Gram-negative bacterium is more resistant than *Staphylococcus*. Compared to acetone and petroleum ether extracts, the methanol extract had greater antibacterial effectiveness, producing the largest inhibitory zone (16 mm at 100 µL). With inhibitory zones of 8 mm and 14 mm at 75 µL and 100 µL, respectively, the acetone extract demonstrated moderate action. The petroleum ether extract showed the least amount of activity, indicating that the non-polar portion contained less active antimicrobial chemicals. For every extract, a dose-dependent rise in the inhibitory zone was seen as concentration increased.

Table 4: MIC for *E.coli*

S.No	Sample ID	MINIMUM INHIBITORY CONCENTRATION – MIC (mm)				MIC of sample (µL)
		<i>E. coli</i>				
		25µL	50µL	75µL	100µL	
1	Methanol Ext.	-	-	08mm	16mm	75µL
2	Acetone Ext.	-	-	08mm	14mm	75µL
3	Petroleum Ether Ext.	-	-	08mm	12mm	75µL

Fig 3: Minimum inhibitory concentrations of *E.coli*

### 3.3 Antifungal Assessment of the *Cajanus cajan* leaf extract

**3.3.1 Zone of inhibition:** Similar antifungal activity was demonstrated by the petroleum ether and methanol extracts, which produced inhibition zones of 12 mm against *Aspergillus* and 10 mm against *Candida*. There were fewer or weaker antifungal chemicals in the acetone extract, as evidenced by its somewhat reduced activity (8 mm and 10 mm). When compared to plant extracts, the conventional medication showed the largest inhibition zones (16 mm against *Candida* and 14 mm against *Aspergillus*), demonstrating its greater antifungal activity. The extracts' overall antifungal efficacy was modest, with *Aspergillus* being more significantly inhibited than *Candida*. The findings imply that bioactive substances with antifungal activity might be extracted using both polar and non-polar solvents.

Table 5: Zone of inhibition for antifungal assay

S.No	Sample Name	Fungal pathogens	
		<i>Candida</i>	<i>Aspergillus</i>
1	Methanol Ext.	10mm	12mm
2	Acetone Ext.	08mm	10mm
3	Petroleum Ether Ext.	10mm	12mm
4	Standard	16mm	14mm

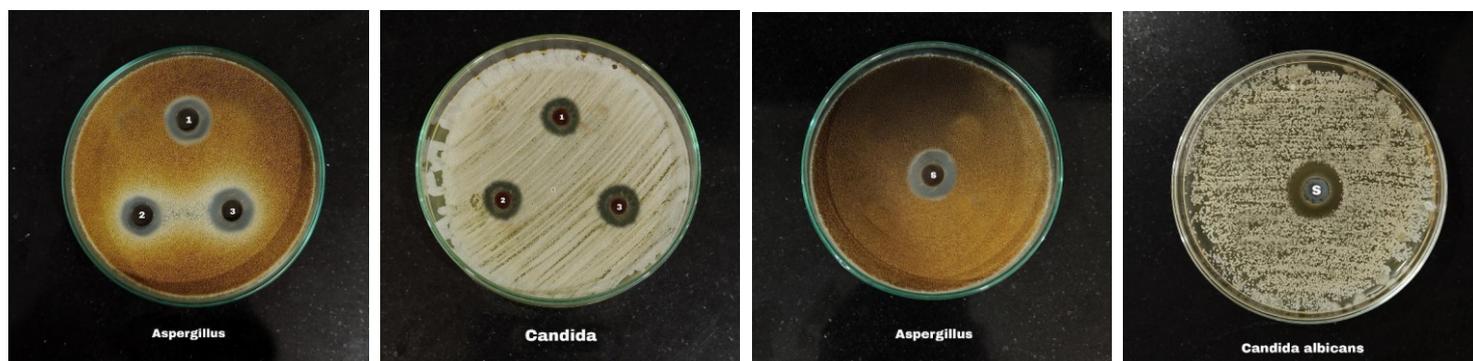
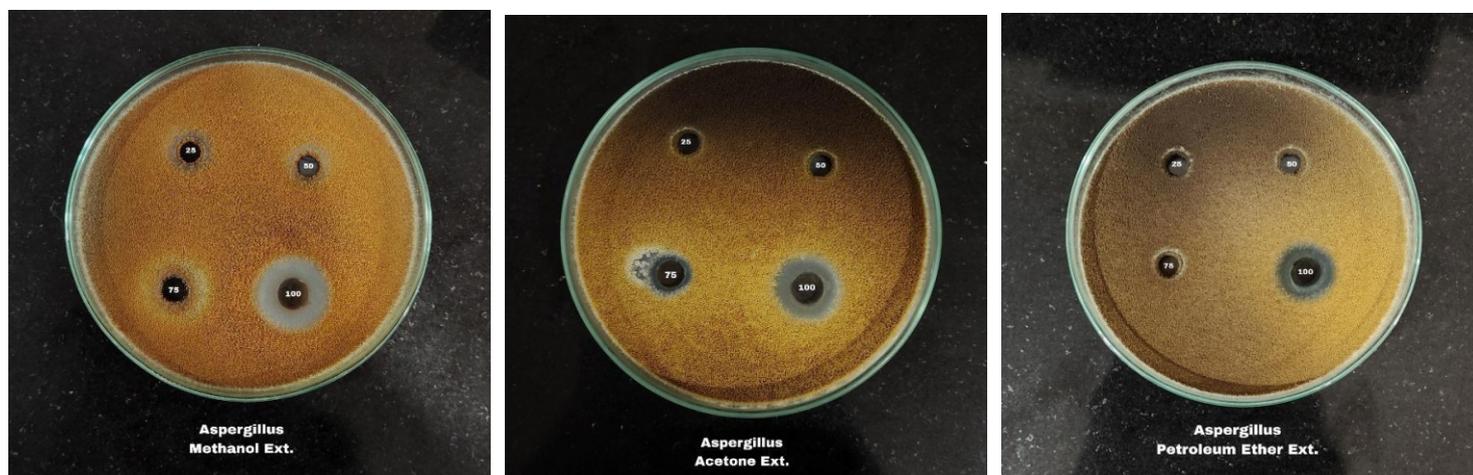


Fig 4: Zone of inhibition of antifungal assay

**3.3.2 Minimum inhibitory concentration (MIC) assay for antibacterial property of given samples:** At lower doses (25–75  $\mu\text{L}$ ), none of the extracts exhibited antifungal action, suggesting that *Aspergillus* was comparatively less sensitive to the tested extracts. Only at 100  $\mu\text{L}$  did all extracts show inhibition, indicating that this is the minimal inhibitory concentration (MIC). Compared to acetone and petroleum ether extracts, the methanol extract had the largest inhibitory zone (16 mm), indicating a potentially higher antifungal effect. With 10 mm inhibition zones, the acetone and petroleum ether extracts had moderate action. The findings show that the antifungal activity is dose-dependent, with greater inhibition at higher doses.

Table 6: MIC for *Aspergillus*

S.No	Sample ID	MINIMUM INHIBITORY CONCENTRATION – MIC (mm)				MIC of sample ( $\mu\text{L}$ )
		<i>Aspergillus</i>				
		25 $\mu\text{L}$	50 $\mu\text{L}$	75 $\mu\text{L}$	100 $\mu\text{L}$	
1	Methanol Ext.	-	-	-	16mm	100 $\mu\text{L}$
2	Acetone Ext.	-	-	-	10mm	100 $\mu\text{L}$
3	Petroleum Ether Ext.	-	-	-	10mm	100 $\mu\text{L}$

Fig 5: Minimum inhibitory concentrations of *Aspergillus*

The methanol extract demonstrated excellent antifungal potential and a distinct dose-dependent response, with inhibitory zones growing from 8 mm to 14 mm as concentration rose, starting at 50  $\mu\text{L}$ . Only at 100  $\mu\text{L}$  did the petroleum ether and acetone extracts show inhibition, indicating relatively lower antifungal activity. The presence of more potent bioactive phytochemicals, most likely polar substances like phenolics, flavonoids, or alkaloids, is indicated by the lower MIC value of methanol extract. Since suppression happened at lower concentrations for at least one extract (methanol), the data also imply that *Candida* is more susceptible to the extracts than *Aspergillus*.

Table 7: MIC for *Candida*

S.No	Sample ID	MINIMUM INHIBITORY CONCENTRATION – MIC (mm)				MIC of sample ( $\mu\text{L}$ )
		<i>Candida</i>				
		25 $\mu\text{L}$	50 $\mu\text{L}$	75 $\mu\text{L}$	100 $\mu\text{L}$	
1	Methanol Ext.	-	08mm	10mm	14mm	50 $\mu\text{L}$
2	Acetone Ext.	-	-	-	10mm	100 $\mu\text{L}$
3	Petroleum Ether Ext.	-	-	-	10mm	100 $\mu\text{L}$

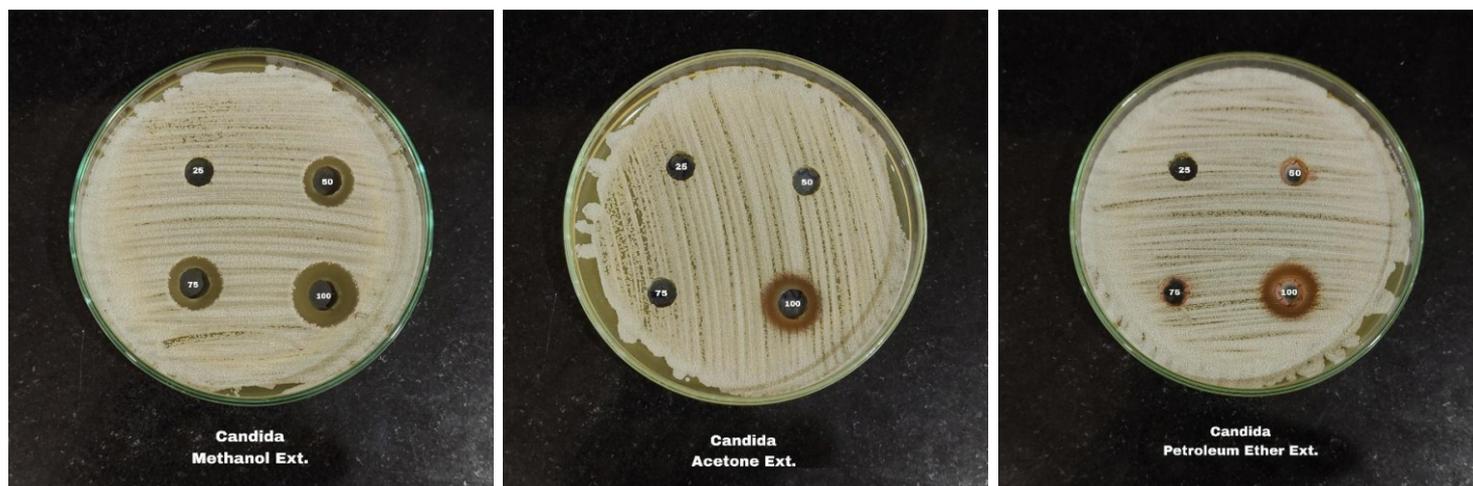


Fig 6: Minimum inhibitory concentrations of *Candida*

#### 4. Conclusion

The current study supported the therapeutic potential of *Cajanus cajan* by showing that leaf extracts made using methanol, acetone, and petroleum ether have varied degrees of antibacterial and antifungal activity. The methanol extract continuously shown the greatest antibacterial activity against both bacterial and fungal infections among the three solvents utilized. This finding suggests that bioactive phyto-constituents with well-known antibacterial qualities, such as phenolics, flavonoids, alkaloids, tannins, and glycosides, are better extracted using polar solvents.

All extracts had inhibitory efficacy against both Gram-positive and Gram-negative bacteria, according to the antibacterial experiments, albeit the degree of inhibition varied according to the concentration and solvent. When compared to acetone and petroleum ether extracts, the methanol extract demonstrated greater antibacterial effectiveness by producing the biggest zones of inhibition and the lowest minimum inhibitory concentration (MIC) values. Gram-negative bacteria have a complex outer layer that prevents antimicrobial chemicals from penetrating them, which may explain their relatively reduced sensitivity. However, the extracts' capacity to inhibit both kinds of bacteria points to a broad-spectrum antibacterial potential.

The extracts' moderate inhibitory activities against fungal diseases were also shown by the antifungal examination. With a lower MIC value than the other extracts, the methanol extract once more demonstrated better action, especially against *Candida*. Higher quantities were needed for acetone and petroleum ether extracts to show detectable inhibition, suggesting that these fractions' antifungal components were comparatively weaker. Due to structural and physiological differences, filamentous fungus may be more resistant to plant-derived chemicals than yeast forms, as indicated by the higher MIC values found against *Aspergillus*.

Overall, the findings support the idea that solvent polarity and concentration have an impact on the antibacterial activity of *Cajanus cajan* leaves. The methanol extract's improved effectiveness emphasizes how crucial solvent selection is for phytochemical extraction and antimicrobial screening investigations. Brito et al. showed that leaf extract was most effective against *Candida albicans* and *Candida tropicalis* [19] and Nwachukwu et al. investigated how *Cajanus cajan* leaf extracts affected *E. coli*, *S. aureus*, *B. subtilis*, and *S. typhi* [20].

Numerous studies have shown that bacteria including *E. coli*, *S. aureus*, *P. gingivalis*, and *S. mutans* can be inhibited in their development by flavonoids (quercetin, genistein, cajanol, etc.) and gallic acid, caffeic acid, etc [21].

In the etiolated stems of *C. cajan*, formononetin has been demonstrated to function as a phytoalexin. Its main function may be as a precursor to the isoflavanone cajanol, which is the main antifungal molecule [22]. The synergistic impact of the extracts' active components is also linked to how well medicinal plant extracts suppress the development of bacteria. The emergence of multi-target mechanisms, the presence of substances that can inhibit bacterial resistance mechanisms, pharmacokinetic or physicochemical effects leading to improved bioavailability, solubility, and resorption rate, neutralization of negative effects, and reduction of toxicity are some of the effects that contribute to the synergistic action [23]. The methanol extract had the most antibacterial action, according to Rokkam et al., especially against *S. aureus* (12.67 mm inhibition), *M. luteus* (19.83 mm inhibition), *S. paratyphi* (12.33 mm inhibition), *S. cerevisiae* (12.50 mm), and *A. niger* (10.63 mm inhibition). Phytochemicals such as phenols, tannins, and flavonoids were shown to be strongly correlated with their antibacterial activities, especially against *B. subtilis*, *S. paratyphi*, and *S. cerevisiae*, according to principal component analysis (PCA) [24].

The results offer scientific support for the traditional usage of the plant in the treatment of illnesses, even if the activity of the plant extracts was lower than that of conventional antibacterial medicines. To sum up, *Cajanus cajan* leaves are a promising natural source of antibacterial chemicals that may find use in the creation of pharmaceuticals. To confirm and improve their therapeutic potential, more research encompassing separation, purification, characterisation of active ingredients, and assessment of toxicity and mechanism of action is advised.

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